

# Lack of Influence of c-Ha-ras Expression on the Drug Sensitivity of Human Bladder Cancer Histocultured in Three-Dimensions

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**Abstract.** *The mechanism of drug resistance in human cancers is complex. In addition to overexpression of a series of multiple-drug-resistance genes, there has been the suggestion that the Ha-ras gene may participate in conferring resistance. In this study, a series of three human-bladder carcinoma cell lines were studied, one parental type, one transfected by wild-type Ha-ras and another transfected by mutant Ha-ras. The ras gene was overexpressed in the latter two cell lines which also were more invasive than the parental when injected as individual cells in the nude-mouse bladder. The results described here have indicated that the ras-gene expression level or mutational status did not affect drug resistance when the tumor lines were histocultured as three-dimensional tissue on collagen-sponge-gels. The drug-response spectrum of the histocultured lines qualitatively reflected a clinical experience although all lines were relatively drug resistant, possibly reflecting their three-dimensional configuration in culture.*

The acquisition of resistance to chemotherapeutic drugs by tumor cells, a well documented clinical phenomenon (1, 2), has been a major obstacle to the effective treatment of cancer. However, the underlying molecular and cellular mechanisms are beginning to be known (3, 4, 5). One mechanism of drug resistance of tumors during treatment appears to involve cellular selection, a process analogous to that of emerging antibiotic resistance in bacteria. Significant populations of drug-resistant cells may be thus inherently produced by clonal evolution of these cells within tumors (6, 7). With respect to such a subclonal evolution, our laboratory has recently demonstrated that metastatically-competent cells, while a cryptic population at the beginning of tumor growth, end up dominating the primary tumor during later

phases (8). In this system the metastatically-competent cells appear to have a new growth advantage over their non-metastatic counterparts which is found to be mediated by paracrine mechanisms (9).

Therefore, if more "malignant" cellular variants are selected from within primary tumors, due to a selective growth advantage, it would appear plausible that these same variants might also be increasingly drug resistant. Such a hypothesis is supported by the following studies. The expression of several oncogenes has been associated with acquisition by cells of a more malignant (10) and in some cases possibly a metastatic phenotype (11) with the c-Ha-ras gene possibly involved (10, 11). It is also interesting to note that the expression of a mutated Ha-ras is involved with radio-resistance (12) and drug resistance (3, 5) in NIH-3T3 fibroblast recipient cells. Thus, Ha-ras may affect several properties essential for the cascade of tumor progression to occur (7).

In the present report we sought to answer two questions. First, does overexpression of mutated H-ras in an already tumorigenic cell line (as opposed to NIH-3T3) confer upon that cell line a more drug-resistant phenotype? Secondly, do more "malignant" cells have an intrinsically greater drug resistance profile, as would be expected by the arguments made above and suggested by Cillo *et al* (13)? To test these two questions we used a bladder carcinoma system in which Ha-ras overexpression seemed to induce orthotopic invasion *in vivo* compared to the non-Ha-ras-transformed parental tumor cell line (10). The drug-resistance profiles of the various cell lines were measured using a three-dimensional sponge-gel-supported histoculture assay (14-21) which simulates *in vivo*-like tumor growth *in vitro*.

Our results show that in this system, neither Ha-ras expression level, Ha-ras mutation status, nor apparent invasive capability influences drug-resistance profiles of transformed cell lines. Our data, therefore, call into question the role of Ha-ras in conferring drug resistance.

## Materials and Methods

*Cell lines.* RT-4 (22), obtained from the American Tissue Culture

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Table I. Drug concentrations and exposure times used in vitro.

Drug	Therapeutic (1 X) concentration	Exposure time, hours
Actinomycin-D	2.0 ng/ml	24
Cisplatin	1.5 µg/ml	24
Mitomycin C	100 ng/ml	24
Vincristine	23 ng/ml	24

The 1 X-drug concentrations and exposure times were calculated from *in vivo* pharmacological data. Suprapharmacologic doses up to 100X were used to compare drug resistance of the bladder-tumor lines described in this report. Drug exposure times are based on the plasma half-life of the various chemotherapeutic agents (38, 39).

Collection (ATCC), is a cell line that was established in 1970 from a 63-year-old patient who had a cystectomy because of a recurrent multifocal, stage-2 grade-1 papillary TCC of the bladder. This line when injected intravesically into athymic "nude" mice behaves like a superficial bladder tumor (23, 10). As described in a recent publication (10), RT-4 was transfected with constructs containing either a 6.4 kb normal cellular *Ha-ras* gene or the 6.6 kb, valine-12-mutated form. Clone RT-4-cr-1, overexpressing the normal *ras*, and clone RT-4-mr-10, overexpressing the mutated *ras*, were found to be invasive when injected as individual cells into the bladder of nude mice as opposed to the parental RT-4 cells which, upon injection, did not invade the nude-mouse bladder wall (10). The transfectant control cell line (RT-4-neo) was generated by transfection of RT-4 with the plasmid pSV2 *neo* (24) and subsequent pooling of >50 G418 resistant colonies.

**Cell culture.** The cell lines were maintained in McCoy's media (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS) under sterile conditions without added antibiotics utilizing standard tissue culture incubators (37° C, 5% CO<sub>2</sub>) and laminar flow hoods. Cells were harvested utilizing 0.25% trypsin, pelleted, washed in medium, repelleted and resuspended in phosphate buffered saline (PBS) in preparation for implantation or passaging.

**Animals.** Female athymic (*nu/nu*) Swiss nude mice were bred in the Animal Care Facility of the Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto. They were first used for the experiments described at 6-8 weeks of age. The animals were maintained in pathogen-free, isolated barrier facilities provided with sterile food, water, bedding and cages.

**Subcutaneous inoculation.** Mice were given injections in the anterior flank with 4 × 10<sup>6</sup> tumor cells of either RT-4-neo, RT-4-cr-1 or RT-4-mr-10 suspended in 0.2 ml of serum-free McCoy's medium. The animals were sacrificed when the tumor became 1.0 cm in diameter.

**Histoculture.** Tumors were explanted and processed as described (14-21). Briefly, the tumors were surgically removed, minced into 2 mm diameter pieces and placed on previously hydrated collagen gel matrices derived from pigskin (Health Design Industries, Rochester, NY). Eagle's minimal essential medium (MEM) containing Earle's salts, glutamine, 10% fetal calf serum, non-essential amino acids and the antibiotic gentamycin were added to culture dishes such that the upper part of the gel was not covered.

**Drug sensitivity assay.** The drugs Actinomycin-D, Mitomycin-C, Cisplatin, and Vincristine were used; their therapeutic concentrations and exposure times are listed in Table I. Immediately after the 24-hour incubation period with the drugs, cell proliferation was measured by administering [<sup>3</sup>H]thymidine (4µCi/ml; 1 Ci = 37° BGq) for 3 days. Cellular DNA is labeled in any cell undergoing replication within the tumor. In the

histoculture assay, [<sup>3</sup>H]thymidine incorporation of human tumors measured by histological autoradiography correlated with drug response of the same tumors growing subcutaneously in nude mice (18). After 3 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules and fixed in 10% formalin. The cultures were then dehydrated, embedded in paraffin and sectioned by standard methodology. After the slides were deparaffinized, they were prepared for autoradiography by coating with Kodak NTB-2 emulsion in the dark, exposed for 5 days, and then the slides were developed. After rinsing, the slides were stained with hematoxylin and eosin. The slides were then analyzed by determining the maximum number of cells undergoing DNA synthesis per 20 × field in treated vs. untreated tumor cultures. Replicating cells were identified by the presence of grains over their nuclei, formed by exposure of the NTB-2 emulsion to radioactive DNA (14-21) the grains were observed as bright green due to the reflection of polarized light.

## Results

Multiple cultures were analyzed for each of the three cell lines. Figure 1 expresses the average percent cell proliferation relative to control for each drug at each of the 4 drug concentrations tested. These concentrations are for mitomycin ( $x = 100$  ng/ml): 10x = 1 µg/ml, 25x = 2.5 µg/ml, 50x = 5 µg/ml, 100x = 10 µg/ml; cisplatin ( $x = 1.5$  µg/ml): 10x = 15 µg/ml, 25x = 37.5 µg/ml, 50x = 75 µg/ml, 100x = 150 µg/ml; actinomycin ( $x=2$  ng/ml): 10x = 20 ng/ml, 25x = 50 ng/ml, 50x = 100 ng/ml, 100x = 200 ng/ml; vincristine ( $x = 23$  ng/ml): 10x = 230 ng/ml, 25x = 575 ng/ml, 50x = 1.15 µg/ml, 100x = 2.3 µg/ml. The latter 3 doses for each drug are considered suprapharmacological (26). It can be seen that for each of the drugs there is no significant difference between the drug sensitivity profiles of the RT-4-cr-1 and the RT-4-mr-10 tumor lines (Figure 1). In addition to this observation, it can be seen that there is virtually no difference in the drug-sensitivity profiles between these two tumor lines and that of the control tumor line, RT-4-neo. This would indicate that in this system, 1) overexpression of the *ras* gene, whether normal or mutated, has no apparent effect on the degree of sensitivity of the cell lines involved since histocultured lines with normal *ras* expression and overexpression of *ras* had a similar drug-response profile, and 2) that the *ras* gene itself may not have any effect in determining drug sensitivity since there was no difference in the drug-response spectrum of histocultured lines with the normal or mutated *ras*. In 3 out of 4 of the drugs tested, the control cell line, RT-4-neo, showed the most resistance even at suprapharmacological doses, which would indicate that the parental cell line is extremely resistant to begin with. The relatively-high drug resistance of all the tumor lines may be due, in part, to the method of culturing used in this study. This resistance may have been overlooked or masked in earlier studies which used monolayer culturing systems. In fact, Hoffman (19) discusses at length some of the recent studies in which it has been shown that many cell lines, when grown in three-dimensional culture systems, show more resistance to the drugs than when they were grown and tested in a monolayer system where there appears to be artificial sensitivity.

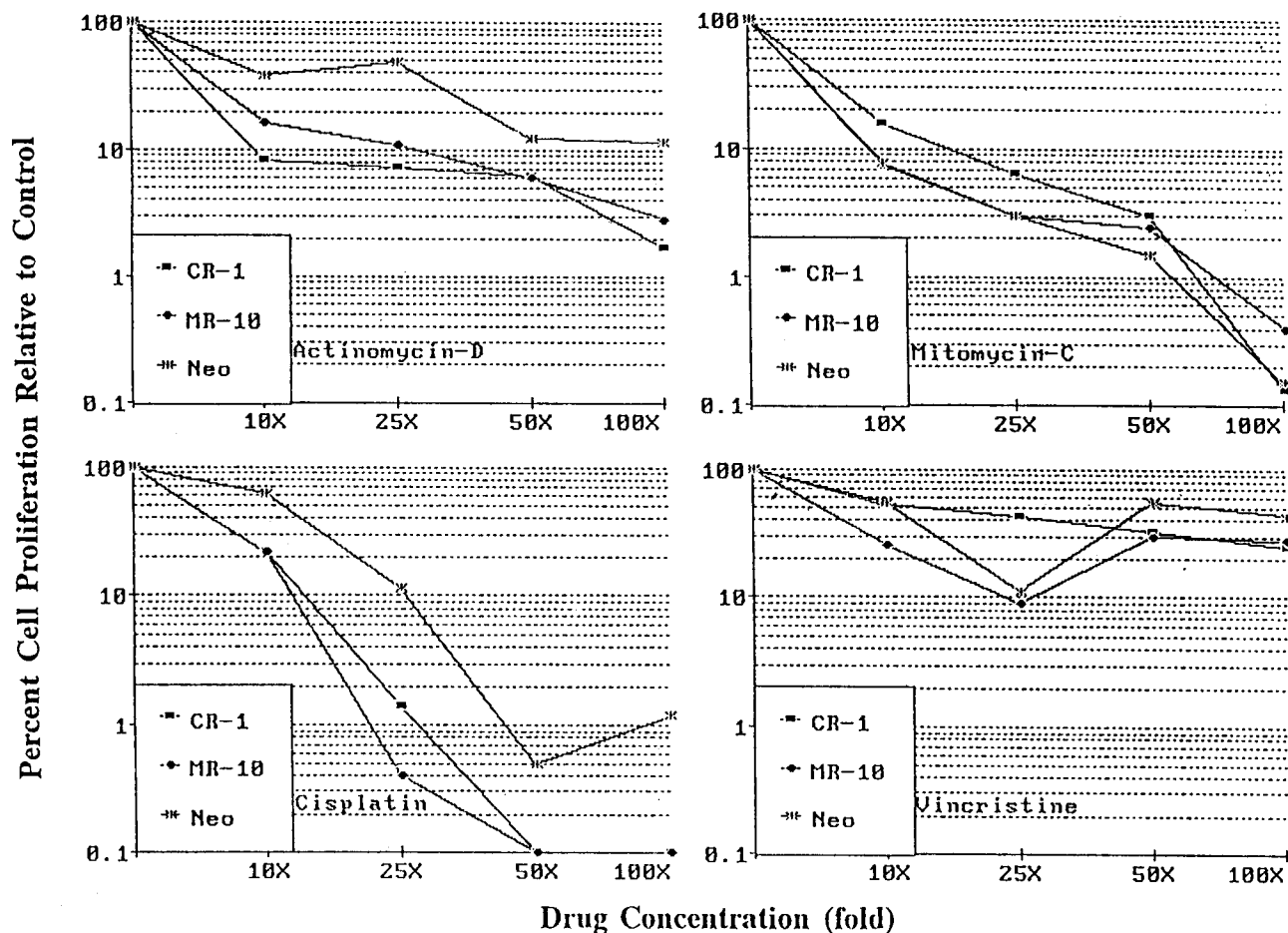


Figure 1. Drug sensitivity profiles for RT-4-cr-1, RT-4-mr-10, and RT-4-nc0 as determined by native-stage 3-dimensional sponge gel histoculture.

In Figure 2, which shows the averaged drug response of the 3 cell lines for each drug, it can be seen that mitomycin (27) and cisplatin (28), two drugs that are known to be highly effective *in vivo*, are also found to be relatively effective in the histoculture assay. On the other hand, actinomycin-D, which is of no clinical value to patients with bladder cancer (29) and was used here as a negative control, is highly ineffective *in vitro*, which is consistent with its *in vivo* profile. Finally, vincristine, which is used in combination chemotherapy of bladder cancer but which has no value as a single agent in this disease (30), is also highly ineffective when used singly in the histoculture assay.

## Discussion

In the above-described experiments we attempted to answer two questions. First, whether the overexpression of *c-Ha-ras* is sufficient to confer drug resistance upon a recipient cell line and second, whether the malignant phenotype is associated

with drug resistance. The first point we attempted to determine in this report is the nature of the relationship between tumor-cell *c-Ha-ras* overexpression and the acquisition of a drug-resistant phenotype. While the studies mentioned above show that NIH-3T3 cells overexpressing a transfected *c-Ha-ras* also acquire a multidrug-resistant phenotype (5) and up-regulate the *mdr-1* and glutathione-S-transferase-P (GST) genes (3), these same cells were also more malignantly transformed during *c-Ha-ras* transfection. Hence, the direct role of *c-Ha-ras* expression on the drug-resistant phenotype could not be evaluated since the transformed phenotypes *per se* could be associated with increased drug resistance. Evidence in support of this is suggested by studies showing that the preneoplastic liver nodules generated by the Solt-Farber model of hepatic carcinogenesis overexpress the *mdr-1* and GST genes (31) and are cross resistant to many chemicals and drugs. In the present study, we have found no relationship between overexpression of either a normal or mutated *c-Ha-ras* in tumorigenic cells and drug resistance. This would

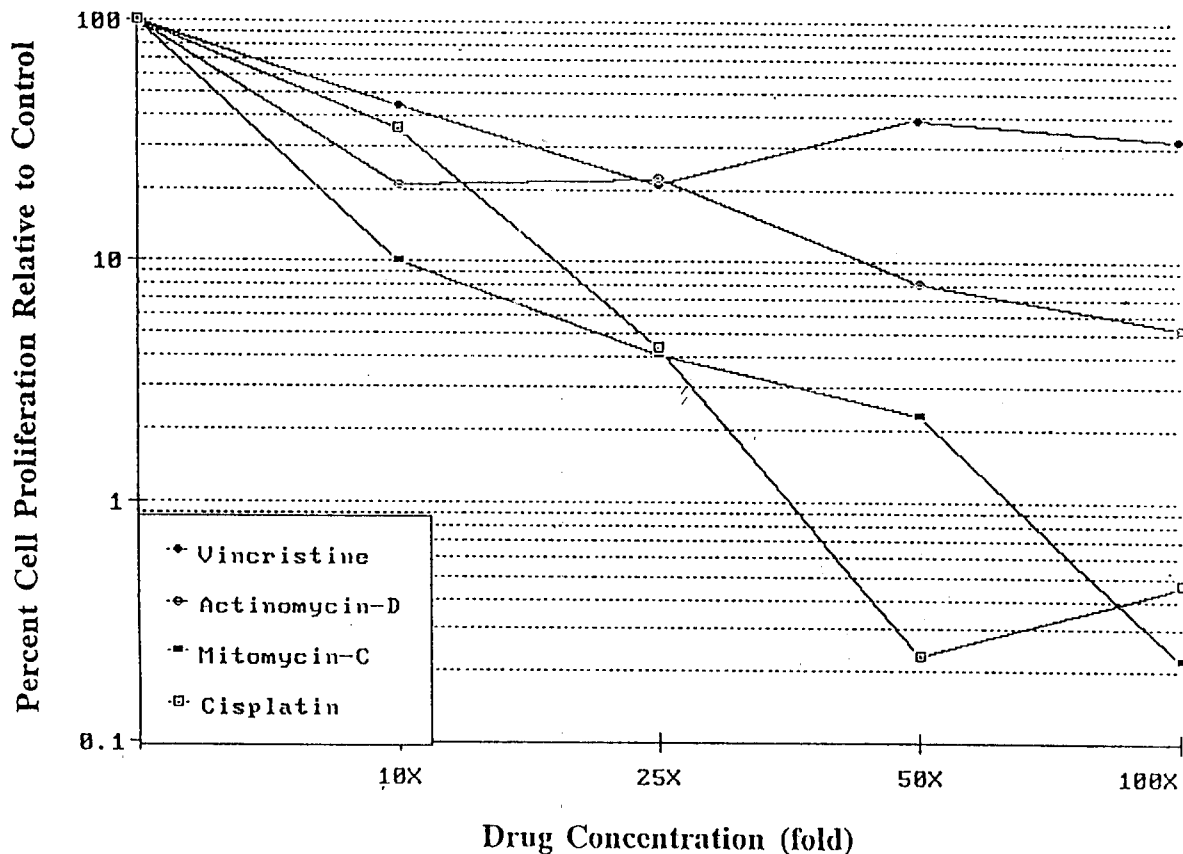


Figure 2. Averaged drug sensitivity profiles for RT-4-cr-1, RT-4-mr-10, and RT-4-neo. Indicating the overall effectiveness of vincristine, actinomycin-D, mitomycin-C, and cisplatin.

strongly suggest that c-Ha-ras expression does not affect the phenotypic drug resistance profiles of cells already transformed.

There are several lines of earlier evidence which have suggested a linkage between increasing tumor aggressiveness and drug resistance. For example, in a series of studies using mouse lymphoma or mouse mammary carcinoma cells of varying metastatic potential, Dennis *et al* (32) discovered a positive correlation between the level of L-PHA binding and relative metastatic aggressiveness. Such an association was also found between L-PHA expression and the level of drug resistance (U. Sagman, unpublished observations). A second line of evidence in support of this hypothesis comes from Poupon's laboratory where *mdr* overexpression variants were selected by chlorozotocin exposure and were subsequently found to be serum independent unlike the parental cells (33). This latter finding is significant since the ability to grow in serum-free medium has been shown to be a phenotypic trait associated with the acquisition of metastatic competence (34) and, therefore, suggested that *mdr* expression correlates with the metastatic phenotype. A more direct approach was taken by Cillo *et al* (13) who found a positive correlation between

the relative metastatic ability of KHT fibrosarcoma cells and their respective drug resistance profiles.

Despite these previous studies, we found no evidence for such a correlation when we tested lineage-related bladder-cancer cell lines of apparently various (*invasive*) potentials (10) in a new *in vivo*-like drug sensitivity assay based on the three-dimensional culture of tissue, termed histoculture (14-21). There may be several explanations for our results. First, our approach involved testing cell lines which possess apparently various invasive abilities in an orthotopic assay system, but which demonstrate similar metastatic potential after intravenous inoculation (10). Thus, metastatic capability measurements seem to be dependent on the nature of the assay system (10). Another significant difference lies in the way that drug sensitivity was measured. The histoculture method used in this study is very different from the classic plating-efficacy methods used in other studies (13). It offers the potential advantage of being a better mimic of the *in vivo* microenvironment during chemotherapeutic treatment of tumors. In addition, the histoculture system as mentioned above supports the growth of tissues in three dimensions which has been shown in a number of studies to confer more

drug resistance than when cells are cultured in two dimensions (19). Differences between *in vivo* and *in vitro* drug resistance have been demonstrated recently (35, 40), highlighting the potential importance of systems such as histoculture used in the present study for the estimation of *in vivo* tumor drug resistance.

These results also demonstrate the validity of the histoculture assay system for the detection of valuable chemotherapeutic drugs for bladder cancer. As can be seen from the results shown in Figure 2, chemotherapeutic agents which are effective in patients with bladder cancer, such as mitomycin C (27) and cisplatin (28), are effective in the histoculture assay. Similarly, agents which are not effective *alone* in bladder cancer chemotherapy such as vincristine (30) and actinomycin-D (29) also faithfully reproduce this behavior in the histoculture assay. Hence, our data and those of others (36, 37) suggest that the histoculture assay and these cell lines may be of value in screening of potentially-useful chemotherapeutic drugs for bladder cancer.

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